VIROLOGY 211, 377–384 (1995)

Microevolution of Type 3 Sabin Strain of Poliovirus in Cell Cultures and Its Implications for Oral Poliovirus Vaccine Quality Control

GENNADY V. REZAPKIN, LAURIE P. NORWOOD, ROLF E. TAFFS, EUGENIA M. DRAGUNSKY, INESSA S. LEVENBOOK, and KONSTANTIN M. CHUMAKOV[1]

Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852

Received April 17, 1995; accepted June 13, 1995

Screening for sequence heterogeneities in Sabin Type 3 strains of attenuated poliovirus demonstrated mutations that consistently accumulate to significant levels following 10 passages in cultures of primary African green monkey kidney (AGMK) cells or continuous cultures of Vero cells. Fourteen newly identified mutations were quantified by mutant analysis by PCR and restriction enzyme cleavage in passages and in batches of commercial vaccines made in AGMK and Vero cells from the Sabin original (SO) seed virus and from a seed virus rederived by RNA plaque purification (RSO or "Pfizer" seed). Nine of the 14 mutations were reproducibly observed in more than one series of passages. Although 5 other mutations were observed in only one set of passages each, their content gradually increased to a high percentage, suggesting that all the mutations that we found accumulated consistently. SO-derived samples accumulated more mutations than did RSO-derived ones, and the number of mutations and the rates of their accumulation were higher in Vero than in AGMK cells. While the rates of accumulation of most mutations were higher when passaging was performed at 37°, a U → C transition at nucleotide 5832 occurred faster at 34°, the temperature used for vaccine production. Analysis of Type 3 oral poliovirus vaccine (OPV) monopools made by six manufacturers found only 5 of these newly identified mutations in vaccine batches (nucleotides 3956, 4935, 5357, 5788, and 5832). Some of the mutations were found in trace amounts (less than 0.1%) while others were present at up to 1.8% levels. The pattern of these mutations was characteristic for the type of seed virus and the cell substrate but demonstrated no correlation with results of the monkey neurovirulence test. Therefore the only mutation occurring in Type 3 OPV which contributed to neurovirulence in monkeys was the previously described reversion at nucleotide 472. Quantitation of reversion at nucleotide 472 can be utilized for assessment of acceptability of vaccine lots, while other mutations can be used for monitoring the consistency of vaccine production.

INTRODUCTION

Oral poliovirus vaccine (OPV) consists of attenuated polioviruses of three serotypes derived by Dr. Albert Sabin from wild-type viruses by rapid serial passaging in primary monkey kidney cell culture at decreased temperature followed by plaque purification of the most attenuated variants (Sabin, 1955, 1957b). Over a period of more than 30 years OPV brought about eradication of poliomyelitis in the western hemisphere and is considered to be the major tool in world-wide eradication of poliomyelitis.

Neurovirulence and attenuation are not discrete features of poliovirus, but rather are quantitative properties varying in a wide range that Dr. Sabin called the "neurotropic spectrum" (Sabin, 1957b). Attenuation can be characterized by a number of phenotypic markers such as rct_{40} (Sabin, 1961) and d-markers (reduced capacity to replicate at higher temperature and at reduced concentration of sodium bicarbonate) and by the lethal or paralytic dose for monkeys. A paralytic dose for the most neurotropic viruses is 1–10 TCD_{50}, whereas inoculation of 10^6–10^7 TCD_{50} of attenuated poliovirus causes no paralysis (Sabin, 1957b). However, the most sensitive tool for evaluating residual neurovirulence of attenuated polioviruses is evaluation and scoring of histological lesions in the central nervous system of rhesus or cynomolgus monkeys after inoculation of the virus into the areas where motor neurons are located (WHO, 1990).

In early studies, it was found that after passaging of attenuated OPV strains in cell cultures or in the intestinal tract of human recipients some quantitative changes in attenuation markers occurred, including changes in rct_{40} and increase in monkey neurovirulence (Sabin, 1957a; Benyesh-Melnick and Melnick, 1959). This gradual deattenuation of the vaccine strains demanded rigorous acceptability testing by the monkey neurovirulence test (MNVT) of every manufactured vaccine monopool before it is used in humans. Despite its high sensitivity and proven reliability, the MNVT has several serious shortcomings including high cost, the need to sacrifice large numbers of primates, and an intrinsic variability of results due to differences in individual reactions of monkeys. An adequate substitute for the MNVT would represent a significant advance in the use of OPV.

Recent work identified genetic and molecular determi-

[1] To whom correspondence and reprint requests should be addressed at CBER/FDA, HFM-255, 1401 Rockville Pike, Rockville, MD 20852. E-mail: CHUMAKOV@HELIX.NIH.GOV.

0042-6822/95 $12.00

nants of neurovirulence for all three type of poliovirus (Evans *et al.*, 1985; Pollard *et al.*, 1989; Kawamura *et al.*, 1989). In Type 3, 2 of the 11 base differences which distinguish Leon $12a_1b$ vaccine strain from its wild-type neurovirulent progenitor Leon/37 were shown to be responsible for the deattenuation occurring in guts of vaccinees (U → C reversions at nucleotides 472 and 2034) (Westrop *et al.*, 1989). We asked whether these reversions occur during vaccine production and contribute to excessive levels of monkey neurovirulence found in some monopools by the MNVT. We developed a sensitive technique, mutant analysis by PCR and restriction enzyme cleavage (MAPREC) and used it to demonstrate that variability at nucleotide 472 is present at a low level in all batches of the vaccine and no reversion at nucleotide 2034 can be found (Chumakov *et al.*, 1991, 1993). The content of 472-C revertants in vaccine batches showed excellent correlation with the histological lesion score in monkeys, and therefore quantitation of revertants at this position could be used to predict results of the MNVT, suggesting that MAPREC might be a suitable alternative or at least a supplement to the monkey test.

Several important questions must be answered before MAPREC can be considered as an adequate replacement for the MNVT. Do any other mutations accumulate during vaccine manufacture and also contribute to deattenuation? Can analysis of one point mutation constitute a reliable alternative to the MNVT? To address these questions we examined genetic changes in the Sabin strain of Type 3 poliovirus after passaging in cell cultures in an attempt to identify other sites in the poliovirus genome where mutations consistently accumulate during vaccine manufacture. We found surprisingly few unstable sites in the genome of Type 3 poliovirus and demonstrated that the mutation profiles of vaccines made in two cell substrates differed. The only consistently occurring mutation that correlated with neurovirulence in monkeys was reversion at nucleotide 472, suggesting that MAPREC assay for this mutation can be used for identification and rejection of vaccine lots with excessive neurovirulence prior to the test in monkeys.

MATERIALS AND METHODS

Cell culture and viruses

Continuous cultures of Vero cells (obtained from ATCC and used at passage levels 144–150) were maintained in MEM with 2 m*M* glutamine and 10% fetal bovine serum. Primary cultures of African green monkey kidney (AGMK) cells (BioWhittaker, Gaithersburg, MD) were used upon receipt without reculturing. When cultures were 95% confluent they were infected with the Sabin strain of poliovirus Type 3 at a multiplicity of infection of about 1–2 TCD_{50} per cell and then incubated at 34 or 37° in medium without serum. Two vaccine monopools of poliovirus Type 3 made by two manufacturers from the Sabin original (SO) seed virus at passage levels SO +2 and SO +3 and two monopools prepared from RNA plaque purified (RSO or "Pfizer") seed at RSO +3 level were used for passaging, which was performed in parallel in two laboratories. After complete cytopathic effects appeared 2–3 days after infection, cultures were frozen and used for further passage and to extract RNA for molecular analysis by sequence heterogeneity assay (SHA) and MAPREC. Ten consecutive virus passages were made in Vero and AGMK cells at each temperature.

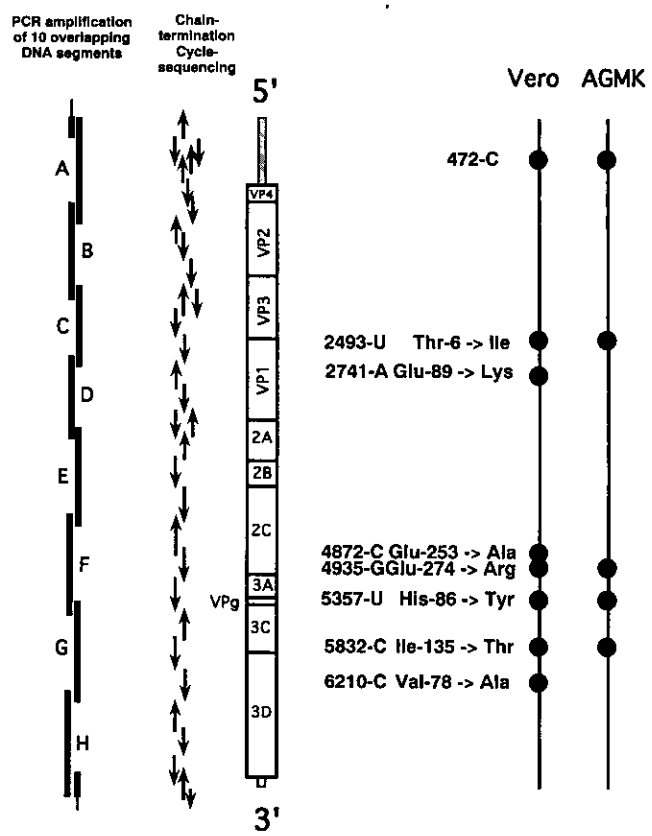

FIG. 1. Type 3 poliovirus genome scheme showing sequencing strategy and mutations consistently accumulating after 10 passages in cell cultures of AGMK and Vero cells.

RNA and cDNA preparation, SHA, and MAPREC

Viral RNA was isolated by phenol/SDS extraction and cDNA was prepared by reverse transcription with Mo-MuLV reverse transcriptase and random hexanucleotide or other primers where indicated. Details of these procedures have been previously described (Chumakov *et al.*, 1991, 1992; Lu *et al.*, 1993; Rezapkin *et al.*, 1994; Taffs *et al.*, 1995). SHA was performed using cycle sequencing of 10 overlapping DNA segments spanning the entire poliovirus genome (Fig. 1), followed by careful inspection of overexposed X-ray films to determine the presence of minor bands indicating possible sequence heterogeneities (Rezapkin *et al.*, 1994). This screening method proved to be very sensitive, in some cases capable of detecting se-

TABLE 1

Primers for MAPREC Assays Used in This Study

Mutation	Enzymes used	Primer names		Sequence	
657 U → C	U cut by *Mse*I	PS-3/655*	621	GAGTTGGATTGGCCATCCAGTGTGAAaCAGATgAA	655
	C cut by *Hin*FI	PA-3/658	692	AACGTTTCGGGAGTGGATCCAACAAACAAGGGAtT	658
940 A → G	A cut by *Alu*I	PS-3/938	904	CGAGCCACTAAAGGACGTGCTCATAAAAACtGCTC	938
	G cut by *Msp*I	PA-3/942*	977	TATACCCACACGCTTCCACATTTGGTGAATTGAGaG	942
1040 U → A		PS-3/1039*	1006	CAATTCCACTATTACTACACAGGAGGCAGCAAgT	1039
	A cut by *Rsa*I	*PA-3/1106*	*1140*	*TAGAATCTGCATGTAGCCACATCTGGTTCAGTTGG*	*1106*
2089 U → C		PS-3/2004	1970	GTTACTCTGAcCGACAGTGCCGATCTATCGCAACC	2004
	C cut by *Dde*I	PA-3/2091*	2126	CACAGAACAGGAAGGTAAATTTCAAGGACCCGGCtC	2091
2150 A → G	A cut by *Tfi*I	PS-3/2149	2115	TCCTGTTCTGTGGTTCAATGATGGCTACGGGGAgA	2149
	G cut by *Ple*I	PA-3/2152*	2180	TGTGCACCTGGTGGTGCATAGGCCACTAGG	2152
2741 G → A	G cut by *Mnl*I	PS-3/2739	2710	CTTCGCACGCGGGGCGTGCGTCGCTATTAT	2739
	A cut by *Mse*I	PA-3/2743*	2773	TGCCCGGGTGGTTGGTTGTTCATTGTCCACC	2743
3956 A → G		PS-3/3955*	3921	CCAGCACGATTACAGAGAAGCTACaTAAAAAaCTA	3955
	G cut by *Spe*I	PA-3/3969	4001	CTTCGTAATTTCTAGTGATAATCACCAGAGATG	3969
4872 A → C	A cut by *Sca*I	PS-3/4870*	4841	GCTTTCGATATGGATATTCAAGTGATGGGC	4870
	C cut by *Hha*I	PA-3/4874	4903	TGCCATGTTGAGTTTACCATCTCTGGAGTA	4874
4935 A → G		PS-3/4930*	4901	GCAATGGCTACTGAGACGTGCAAGGACTGC	4930
	G cut by *Mbo*I	PA-3/4936	4970	ACACTAAAGGACAGCATCTTTTGAAGTTTGCTGaT	4936
5357 C → U		PS-3/5356*	5321	GGTGTCGTGTACGTCATGTACAAGTTATTCGaTcGA	5356
	U cut by *Cla*I	PA-3/5367	5400	ACATTGGGTCTTTTGTTTGGCAGACCAGTGTATG	5367
5788 U → C		PS-3/5786*	5754	ACCCCAACATGTATGTTCCTGTCGGTGCTGgGA	5786
	C cut by *Ava*II	PA-3/5849	5880	ATGACTCCACCACACTGACCAGCTCTGGTTGG	5849
5832 U → C		PS-3/5758	5726	GGAGTTCTGATTGTGAACACTAGTAAGTAgCCC	5758
	C cut by *Rsa*I	PA-3/5833*	5870	CACACTGACCAGCTCTGGTTGGAAAGTTGTACATTcGA	5833
6210 U → C	U cut by *Bsr*I	PS-3/6208*	6174	AGATCACTGAGGTGGATGAGTACATGAAAGAGcCA	6208
	C cut by *Msp*AI	PA-3/6212	6241	CAGCGACATAAGTTGTCCAGCATAATGGTC	6212
6979 U → C		PS-3/6978*	6941	AAATGATTGCCTATGGTGACGATGTAATAGCTTCgTA	6978
	C cut by *Rsa*I	PA-3/7038	7073	CTGTCTCAAAAGTGGCAGATTTATCTGCCGGAGTCA	7038

Note. Lowercase letters indicate differences from the sequence of Type 3 poliovirus RNA introduced to create or eliminate restriction sites.
* Radiolabeled primer.

quence heterogeneity below the level of 1%. Segments including 5′- and 3′-termini of cDNA were made using synthetic template extension oligonucleotides (Rezapkin *et al.*, 1994). Each site identified by SHA was independently verified and quantified by MAPREC (Chumakov *et al.*, 1991; Lu *et al.*, 1993). Primers and restriction enzymes used for MAPREC assays are listed in Table 1. Restriction digests of radiolabeled PCR products were separated by polyacrylamide gel electrophoresis for quantitation in a Betascope B603 blot analyzer (Betagen). To evaluate quantitative performance and specificity of MAPREC we included both a negative control (cDNA plasmid containing an entire copy of the Sabin 3 poliovirus kindly provided by Dr. A. Macadam, NIBSC, UK) and a positive control (cDNA of a revertant virus or DNA made by PCR *with oligonucleotide primer modified to create reversion*) in each test. In the majority of cases MAPREC was extremely sensitive and we could easily quantitate mutations at a level of 0.1% or even less. In some cases determination of very low amounts of mutants was not as sensitive (nucleotide 5357 with a sensitivity limit about 0.5% and nucleotides 940, 2150, 2741, 3956, and 5788 with a sensitivity limit about 0.2%), for several reasons: (i) presence of revertant-specific digestion products in the plasmid control, suggesting nucleotide misincorporation during PCR, which sets a lower limit on sensitivity of MAPREC (Lu *et al.*, 1993); (ii) high background radioactivity smeared along the polyacrylamide gel lanes, making quantitation of small bands less precise; and (iii) presence of artifactual DNA products (e.g., primer–dimers) interfering with determination of revertant-specific digestion products. Therefore for some nucleotide positions we were able to determine the quantity of mutants precisely and for others we could only see that the level of revertants in OPV lots was not higher than the apparent level detected in the cDNA plasmid. In the latter case we indicate the lower limit of detection of the particular MAPREC test.

RESULTS

Sequence heterogeneities in high-passage samples of Type 3 Sabin strain of poliovirus

To identify mutations that consistently accumulate in the virus genome we serially passaged two monovalent batches of SO-derived vaccine and two batches of RSO-derived vaccine from different manufacturers in AGMK

TABLE 2

Mutations Detected in the Sabin Strain of Type 3 Poliovirus after 10 Passages in Cell Culture

				AGMK						Vero			
				34°				37°		34°		37°	
				RSO		SO							
Site	Mutation	Amino acid	Gene	Expt 1	Expt 2	Expt 1	Expt 2	RSO	SO	RSO	SO	RSO	SO
657	T → C	—	NTR									39%	
940	A → G	Silent	VP4										69%
1040	T → A	Ser → Thr 31	VP2		15%								
2089	T → C	Silent	VP3									94%	
2150	A → G	Ile → Val 130	VP3				23%						
2741	G → A	Glu → Lys 89	VP1					<1%	<1%	<1%	1%	95%	91%
3956	A → G	Ile → Val 12	2B			3%	1%		3%		<1%		8%
4872	A → C	Glu → Ala 253	2C			5%	<1%		1%	52%	25%	27%	88%
4935	A → G	Gln → Arg 274	2C	1%		16%	4%	3%	12%		1%	65%	6%
5357	C → T	His → Tyr 86	3A	2%	7%	7%	14%	16%	25%	68%	16%	80%	85%
5788	T → C	Silent	3C pro			13%	31%		31%		1%		2%
5832	T → C	Ile → Thr 135	3C pro	60%	44%	36%	7%	3%	3%	50%	70%	1%	1%
6210	T → C	Val → Ala 78	3D pol					<1%				63%	
6979	T → C	Silent	3D pol			7%	20%		20%		1%		2%

and Vero cells at 34° (temperature used for vaccine manufacture) and 37° (supraoptimal temperature favoring reversions) to allow mutations to accumulate to a detectable level. Ten high-passage samples were prepared, including samples independently passaged in AGMK cultures in two laboratories. After 10 passages, samples were analyzed by SHA (see Materials and Methods) and 14 new mutations were identified in addition to reversions at nucleotides 472 and 2493 previously described (Evans *et al.*, 1985; Weeks-Levy *et al.*, 1991; Chumakov *et al.*, 1993). MAPREC assays were designed for each of these mutations and the results of their quantitation in high-passage samples are given in Table 2. Nine of 14 newly identified mutations were reproducibly found under more than one cell culture condition. Mutations consistently accumulating in AGMK and Vero cells are presented on Fig. 1. Analysis of two pairs of SO and RSO viruses passaged independently in AGMK cells demonstrated remarkable similarity of patterns of these mutations. Five mutations that were found only in a single set of cell culture conditions (657-C, 940-G, 1040-A, 2089-C, and 2150-G) gradually increased to high percentages (Fig. 2), suggesting that they may also have accumulated consistently and are not simply the result of a "bottleneck" phenomenon (the random change in a population structure caused by nonrepresentative composition of a small inoculum).

Mutations presented in Table 2 and Fig. 1 show different patterns of accumulation and can be classified into several groups. 3956-G, 5788-C, and 6979-C were found only in SO-derived samples. 2741-A, 4872-C, and 6210-C accumulated almost exclusively in Vero cells and were found in AGMK-passaged virus only at very low levels. 4935-G and 5257-T accumulated up to 25% in AGMK-passaged virus and up to 85% in Vero-passaged virus. 5832-C was found in all the samples and, unlike other mutations, accumulated faster at the lower (34°) temperature.

OPV lots obtained from six manufacturers were screened using MAPREC for mutations observed in the laboratory cultures. Five of the nine mutations that accumulated reproducibly in cell culture passages were detected in vaccine lots. Table 3 shows the content of mutations in OPV lots at these sites and at nucleotides 472 and 2493 previously studied (Chumakov *et al.*, 1992,

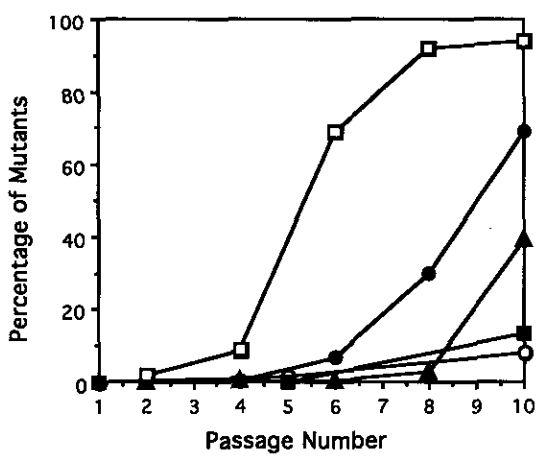

FIG. 2. Accumulation of mutants in the Sabin 3 poliovirus passaged in AGMK and Vero cells. ▲, 657 T → C in Vero cells at 37°; ●, 940 A → G in Vero cells at 37°; ○, 1040 T → A in AGMK cells at 34°; □, 2089 T → C in Vero cells at 37°; ■, 2150 A → G in AGMK cells at 34°.

TABLE 3

Mutations Found in Monopools of Type 3 OPV Made by Six Manufacturers (A–F)

Manufacturer and lot number (coded)	Percentage of mutants						
	472-C	2493-U	3956-G	4935-G	5357-U	5788-C	5832-C
Plasmid control	0.10	0.00	0.13	0.03	0.52	0.10	0.00
	Monopools that have failed the MNVT (SO-derived)						
A1	2.22	86	0.64	0.19	—	0.24	0.22
B1	1.64	28	1.15	0.73	—	—	0.08
C1	1.32	84	0.74	0.27	—	—	0.07
	Monopools that have passed the MNVT						
SO-derived monopools							
US reference	0.85	33	0.69	0.39	—	—	0.10
WHO reference	0.28	55	0.57	0.06	—	—	0.04
B2	0.62	33	1.05	0.48	—	—	0.07
D1	0.51	58	0.82	0.10	—	—	0.09
E1	0.75	15	1.16	0.48	—	0.19	0.16
E2	0.69	37	1.30	0.54	—	0.12	0.24
RSO-derived monopools							
B3	0.47	1.92	—	—	—	—	0.03
B4	0.55	1.60	—	—	—	—	0.05
A2	0.29	0.76	—	—	—	0.12	0.15
A3	0.38	0.59	—	—	—	—	0.02
A4	0.42	0.48	—	—	—	—	0.07
D2	0.26	0.25	—	—	—	—	0.15
RSO-derived monopools made in Vero cells							
F1	0.48	1.10	—	0.41	1.89	—	0.29
F2	0.55	1.40	—	0.32	0.97	—	0.31

Note. Dash indicates that mutation was not found in quantitites exceeding the background determined in homogenous control (cDNA plasmid).

1994). Vaccines made from SO seed virus contained more sequence heterogeneities, including 3956-G and 4935-G, which were only observed in samples derived from cultures infected with the SO strain (see above). 5832-C, which accumulated predominantly at 34°, was present at a relatively low level in all vaccine lots.

RSO-derived vaccine lots made in AGMK and Vero cells had different patterns of mutations. Two batches of Type 3 OPV made in Vero cells contained 5357-U, which also accumulated predominantly in the virus serially passaged in Vero cells. 4935-G was found both in SO-derived vaccine batches produced in AGMK and in RSO-derived monopools made in Vero cells.

DISCUSSION

The low fidelity of poliovirus RNA replication, with characteristic error rates of 10^{-3}–10^{-4} (Ward *et al.*, 1988), results in emergence of a variety of mutations randomly scattered along the viral genome (Ward and Flanegan, 1992). Therefore polioviruses, like other RNA viruses, constitute quasispecies — populations of viral particles with a variety of genomic sequences differing slightly from a consensus sequence (Holland *et al.*, 1982; Domingo *et al.*, 1985). Lethal and other strongly deleterious mutations are rapidly eliminated, while selectively neutral and positive mutations persist in a population. At such high mutation rates, a single dose of OPV is predicted to contain hundreds of copies of each possible point mutation and many combinations of two point mutations. Therefore, strictly speaking, molecular control of OPV is not a matter of detecting the presence in vaccine batches of mutants with increased neurovirulence, but rather a quantitative discrimination between batches with acceptable and unacceptable levels of these mutations. Recently we introduced the MAPREC method for quantitation of mutations in samples of OPV and have demonstrated that (i) all batches of Type 3 OPV contain detectable levels of a neurovirulent reversion at nucleotide 472, and (ii) the content of 472-C was higher in batches that failed the MNVT (Chumakov *et al.*, 1991). Quantitative determination of 472-C in Type 3 OPV proved to have good predictive power for the outcome of the MNVT and can be considered as an alternative to the latter (Chumakov *et al.*, 1993). In contrast, 2493-U mutation which is also present in all batches of Type 3 OPV and rapidly accumulates during the virus growth in cell cultures, did not show correlation with neurovirulence in monkeys but could be used to determine the seed virus type and the passage level of the vaccine (Chumakov *et*

al., 1992, 1993). In the present study we attempted to identify other sites in Type 3 poliovirus genome that may also contain mutations. For the reasons discussed above, we did not try to identify neutral mutations that are present in every vaccine batch at a low background level, but rather concentrated on finding selectively positive mutations that accumulate by giving the virus replicative advantage under the conditions of vaccine manufacture. A similar approach was recently used in our studies of OPV Types 1 and 2 (Rezapkin *et al.,* 1994; Taffs *et al.,* 1995).

The result of this screening was a relatively short list of genomic sites at which sequence heterogeneities appeared by the 10th passage. It is important to note that most of these heterogeneities were observed in more than one passaging lineage (in different cell cultures, at different temperatures, or in different substrains of the Sabin 3 poliovirus), which argues against the incidental nature of these mutations. In cases where mutations were identified in only one set of conditions, their accumulation in passages also followed a consistent pattern.

Mutations that were identified in this study can be separated into several groups. Three mutations (3956-G, 5788-C, and 6979-C) were strictly seed virus-specific and were found only in passages derived from the SO seed. Analysis of batches of vaccine confirmed that 3956-G and 5788-C were detectable in SO-derived lots of OPV, while plaque-purified RSO seed virus produced more homogeneous vaccine batches.

The optimal temperature for growth of attenuated poliovirus is 34°, and upon replication at increased temperature (37°) the virus deattenuates much faster. This observation correlates with our finding that most of the mutations accumulated faster at 37° than at 34°. There was one notable exception, a mutation at nucleotide 5832 which accumulated rapidly at 34° and very slowly at 37°. 5832-C was universally present in vaccine monopools, albeit at very low levels.

Another general trend, which is also true for attenuated polioviruses of Types 1 and 2, is that a higher number of mutations with higher rates of accumulation occurred when Type 3 poliovirus was passaged in Vero cells. The Sabin strains were derived in primary cultures of monkey kidney cells, and the strains seem to be better adapted to this substrate. Switching to a different cell substrate may require the virus to change. Analysis of two vaccine batches produced in Vero cells showed that they shared a distinct pattern of mutation. Acceptable results of the MNVT for Vero-produced vaccines argue against the role of these mutations in neurovirulence, at least at the levels of abundance present. However, higher rates of accumulation for mutations in poliovirus propagated in Vero cells calls for added caution and demands that the role of these mutations in neurovirulence be studied in experiments with point mutation constructs. This also applies to other mutations, especially to 5832-C.

Similar to the situation with Types 1 and 2, selectable mutations in Type 3 fall into three categories: mutations in untranslated regions, missense mutations, and silent mutations. Two of the silent mutations are specific for the SO seed virus and accumulated up to 30% in passages. Two other silent mutations (940-G and 2089-C) accumulate only in Vero cells at 37° to levels about 70–90%. Accumulation of silent mutations was previously described by others (De La Torre *et al.,* 1992; Borzakian *et al.,* 1993) as well as by us in polioviruses of Types 1 and 2 (Rezapkin *et al.,* 1994; Taffs *et al.,* 1995). "Biased hypermutation" by RNA editing or some other mechanism was proposed to explain occurrence of multiple silent mutations on single RNA molecules (De La Torre *et al.,* 1992). In our case, the mechanism responsible for accumulation of silent mutations is unknown and may involve codon preference or alteration of RNA secondary structure that directly participates in some important replication process. Another mechanism for selection of silent mutations would be a "passenger" effect, i.e., mutant selection driven by another mutation with a strong selective advantage that occurred incidentally on the same RNA molecule. The passenger effect seems likely to have selected those silent mutations that accumulated in only one passaging lineage, e.g., 940-G and 2089-C, which are accompanied by other highly selectable mutations. This possibility can be tested in experiments with single point mutations.

It is easier to suggest a physiological role for missense mutations, even though some of them may also be neutral passengers. Good candidates for this latter category are mutations that accumulate consistently, but only in passages derived from the SO substrain, and therefore may have been present in the original SO population. At least two such mutations were found in SO but not in RSO vaccines.

Mutations that occurred in both SO and RSO substrains of the Sabin 3 poliovirus are most likely to be genuine positively selectable mutations. It should be mentioned that the phenotypic effects of all newly identified mutations may not be very strong, causing only subtle changes of the viral replicative capacity. A G-to-A mutation at nucleotide 2741 occurred in both SO and RSO virus stocks. It demonstrated strong cell–substrate specificity, accumulating in Vero cells to almost complete substitution, while in AGMK cells it was present at less than 1% level after 10 passages. This mutation changes glutamic acid to lysine at amino acid 89 in a β-strand B of a VP1 protein core and therefore can be expected to produce a change in its structure. It is noteworthy that mutation at the same location was found in Type 1 poliovirus after replication in cell culture at elevated temperature (Christodoulou *et al.,* 1990) and in the central nervous system of monkeys (our unpublished observation).

Two missense mutations in the 2C protein, Glu253 → Ala (nucleotide 4872) and Gln274 → Arg (nucleotide

4935), accumulated mostly in Vero cells, and to a much lesser degree in AGMK cells. These almost ubiquitous mutations are located next to the region of the 2C molecule where guanidine-resistant and ts RNA$^-$ mutations occur (Li and Baltimore, 1988; Baltera and Tershak, 1989), suggesting that they may also affect viral RNA synthesis. Previously we found that Gln274 → Arg mutation also accumulates in polioviruses Types 1 and 2 passaged in Vero cells (Rezapkin *et al.*, 1994; Taffs *et al.*, 1995). Therefore it seems to be responsible for the ability of all three types of poliovirus to replicate in Vero cells, suggesting that 2C may specifically interact with a cellular component which is different in Vero and AGMK cells. It is noteworthy that host-dependent mutations in the 2C protein have been also identified in hepatitis A virus (Emerson *et al.*, 1991, 1992; Graff *et al.*, 1994).

A missense mutation at nucleotide 5357, changing His to Tyr at penultimate amino acid 86 of the 3A protein, was identified in all 10 independent passaging lineages. In some cases it substituted up to 80–85% of the original nucleotide. Its role should also be studied in experiments with point mutation constructs, but its location in the immediate vicinity of the 3A/VPg junction suggests that it may be involved in initiation of RNA synthesis. Insertion mutants at this site were found to be lethal (Kuhn *et al.*, 1988).

Perhaps the most important question is whether these newly identified mutations increase neurovirulence of attenuated poliovirus. Some information can be obtained by analysis of vaccine monopools that either passed or failed the neurovirulence test in monkeys. The only mutation that shows a clear correlation with results of the MNVT is reversion at nucleotide 472, while other mutations (like 2493-U discussed above) may still be used to identify the seed virus type and monitor consistency of vaccine production. It is noteworthy that two batches of vaccines made in Vero cells contained an additional mutation at nucleotide 5357 which was universally selected in passages under all conditions, but increased much faster and to higher levels in Vero cells. However, this mutation could not be found in any AGMK-derived vaccine lots. In addition, another mutation with a similar pattern of accumulation (4935-G) was found both in SO-derived vaccines and in RSO-derived vaccines manufactured in Vero cells. Thus mutation profile of vaccines produced in Vero cells is distinct from that of AGMK-produced vaccines. It remains to be seen whether these mutations affect neurovirulence of the vaccine.

In this study we performed fine molecular genetic analysis of populations of attenuated Type 3 poliovirus. Apart from better understanding of details of the molecular evolution of poliovirus quasispecies, this work has practical implications. The first one is related to our efforts to develop a molecular test for assessing the genetic stability of OPV and other live vaccines by analyzing the content of mutations critical to maintaining an attenuated phenotype. In this respect it is clear that reversion at nucleotide 472 should be tested as a neurovirulence marker of Type 3 poliovirus. Whether any of the other mutations identified in this study should also be tested remains to be established.

A second practical implication of this work is that it suggests the possibility of creating genetically refined strains of OPV. Mutations that do not contribute to a virulent phenotype, but which are selected and provide replicative advantages, might be introduced into the vaccine virus genome to boost the yield of OPV, improve its potency, and stabilize its genome. As we discussed above, the passenger effect may result in accumulation of incidental mutations even though they have no replicative advantage. Some of these incidental mutations may have undesired properties. Introduction of mutations that boost virus replication without affecting attenuation would eliminate the driving force for changes in the quasispecies population and result in more homogeneous, genetically stable, and safer vaccines. Such genetic refinement may become a general approach applicable to other live viral vaccines.

ACKNOWLEDGMENTS

Authors thank Dr. Yuri Svitkin for providing the high-passage samples of poliovirus and Dr. Edward Fitzgerald, Dr. Ronald Lundquist, and Dr. David Asher for discussions and critical review of the manuscript.

REFERENCES

Baltera, R. F., and Tershak, D. R. (1989). Guanidine-resistant mutants of poliovirus have distinct mutations in peptide 2C. *J. Virol.* **63**, 4441–4444.

Benyesh-Melnick, M., and Melnick, J. L. (1959). The use of *in vitro* markers and monkey neurovirulence tests to follow genetic changes in attenuated poliovirus multiplying in the human alimentary tract. *Pan-American Health Organization, Sci. Pub. No. 44*, pp. 179–198. Washington, DC.

Borzakian, S., Pelletier, I., Calvez, V., and Colbere-Garapin, F. (1993). Precise missense and silent point mutations are fixed in the genomes of poliovirus mutants from persistently infected cells. *J. Virol.* **67**, 2914–2917.

Christodoulou, C., Colber-Garapin, F., Macadam, A., Taffs, L. F., Marsden, S., Minor, P., and Horaud, F. (1990). Mapping of mutations associated with neurovirulence in monkeys infected with Sabin 1 poliovirus revertants selected at high temperature. *J. Virol.* **64**, 4922–4929.

Chumakov, K. M. (1994). Reverse transcriptase can inhibit PCR and stimulate primer-dimer formation. *PCR Methods Appl.* **4**, 62–64.

Chumakov, K. M., Dragunsky, E. M., Norwood, L. P., Douthitt, M. P., Ran, Y., Taffs, R. E., Ridge, J., and Levenbook, I. S. (1994). Consistent selection of mutations in the 5′-untranslated region of oral poliovirus vaccine upon passaging *in vitro*. *J. Med. Virol.* **42**, 79–85.

Chumakov, K. M., Norwood, L. P., Parker, M. L., Dragunsky, E. M., Ran, Y., and Levenbook, I. S. (1992). RNA sequence variants in live poliovirus vaccine and their relation to neurovirulence. *J. Virol.* **66**, 966–970.

Chumakov, K., Norwood, L., Parker, M., Dragunsky, E., Taffs, R., Ran, Y., Ridge, J., and Levenbook, I. (1993). Assessment of the viral RNA sequence heterogeneity for control of OPV neurovirulence. *Dev. Biol. Stand.* **78**, 79–89.

Chumakov, K. M., Powers, L. B., Noonan, K. E., Roninson, I. B., and

Levenbook, I. S. (1991). Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. *Proc. Natl. Acad. Sci. USA* **88,** 199–203.

De La Torre, J. C., Giachetti, C., Semler, B., and Holland, J. J. (1992). High frequency of single-base transitions and extreme frequency of precise multiple-base reversion mutations in poliovirus. *Proc. Natl. Acad. Sci. USA* **89,** 2531–2535.

Domingo, E., Martinez-Salas, E., Sobrino, F., De La Torre, J. C., Portela, A., Ortin, J., Lopez-Golindez, C., Perez-Brena, P., Villa-Nueva, N., Najera, R., VandePol, S., Steinhauer, D., DePolo, N., and Holland, J. J. (1985). The quasispecies (extremely heterogenous) nature of viral RNA genome populations: Biological relevance—A review. *Gene* **40,** 1–8.

Emerson, S. U., McRill, C., Rosenblum, B., Feinstone, S., and Purcell, R. H. (1991). Mutations responsible for adaptation of hepatitis A virus to efficient growth in cell culture. *J. Virol.* **65,** 4882–4886.

Emerson, S. U., Huang, Y. K., McRill, C., Lewis, M., and Purcell, R. H. (1992). Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. *J. Virol.* **66,** 650–654.

Evans, D. M. A., Dunn, G., Minor, P. D., Schild, G. C., Cann, A. J., Stanway, G., Almond, J. W., Currey, K., and Maizel, J. V. (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature* **314,** 548–550.

Graff, J., Normann, A., Feinstone, S. M., and Flehmig, B. (1994). Nucleotide sequence of wild-type hepatitis A virus GBM in comparison with two cell culture-adapted variants. *J. Virol.* **68,** 548–554.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., and VandePol, S. (1982). Rapid evolution of RNA genomes. *Science* **215,** 1576–1585.

Kawamura, N., Kohara, M., Abe, S., Komatsu, T., Tago, K., Arita, M., and Nomoto, A. (1989). Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. *J. Virol.* **63,** 1302–1309.

Kuhn, R. J., Tada, H., Ypma-Wong, M. F., Semler, B. L., and Wimmer, E. (1988). Mutational analysis of the genome-linked protein Vpg of poliovirus. *J. Virol.* **62,** 4207–4215.

Li, J.-P., and Baltimore, D. (1988). Isolation of poliovirus 2C mutants defective in viral RNA synthesis. *J. Virol.* **62,** 4016–4021.

Lu, Z., Douthitt, M. P., Taffs, R. E., Ran, Y., Norwood, L. P., and Chumakov, K. M. (1993). Quantitative aspects of the mutant analysis by PCR and restriction enzyme cleavage (MAPREC). *PCR Methods Appl.* **3,** 176–180.

Pollard, S. R., Dunn, G., Cammack, N., Minor, P. D., and Almond, J. W. (1989). Nucleotide sequence of a neurovirulent variant of the Type 2 oral poliovirus vaccine. *J. Virol.* **63,** 4949–4951.

Rezapkin, G. V., Chumakov, K. M., Lu, Z., Ran, Y., Dragunsky, E. M., and Levenbook, I. S. (1994). Microevolution of attenuated poliovirus *in vitro* and genetic stability of oral poliovirus vaccine. *Virology* **202,** 370–378.

Sabin, A. B. (1955). Characteristics and genetic potentialities of experimentally produced and naturally occurring variants of poliomyelitis virus. *Ann. N.Y. Acad. Sci.* **61,** 924–938.

Sabin, A. B. (1957a). Properties of attenuated polioviruses and their behavior in human beings. *In* "Special Publication of the New York Academy of Sciences," Vol. V, pp. 113–127. N.Y. Acad. Sci., New York.

Sabin, A. B. (1957b). Properties and behavior of orally administered attenuated poliovirus vaccine. *JAMA* **164,** 1216–1223.

Sabin, A. B. (1961). Reproductive capacity of polioviruses of diverse origin at various temperatures. *In* "Perspectives in Virology" (M. Pollard, Ed.), Vol. 2, pp. 90–108. Burgess, Minneapolis.

Taffs, R. E., Chumakov, K. M., Rezapkin, G. V., Lu, Z., Douthitt, M., Dragunsky, E. M., and Levenbook, I. S. (1995). Genetic stability and mutant selection in Sabin 2 strain of oral poliovirus vaccine grown under different cell culture conditions. *Virology* **209,** 366–373.

Ward, C. D., and Flanegan, J. B. (1992). Determination of the poliovirus RNA polymerase error frequency at eight sites in the viral genome. *J. Virol.* **66,** 3784–3793.

Ward, C. D., Stokes, M. A. M., and Flanegan, J. B. (1988). Direct measurement of the poliovirus RNA polymerase error frequency *in vitro*. *J. Virol.* **62,** 558–562.

Weeks-Levy, C., Tatem, J. M., DiMichele, S. J., Waterfield, W., Georgiu, A. F., and Mento, S. J. (1991). Identification and characterization of a new base substitution in the vaccine strain of Sabin 3 poliovirus. *Virology* **185,** 934–937.

Westrop, G. D., Wareham, K. A., Davis, D. M. A., Dunn, G., Minor, P. D., Magrath, D. I., Taffs, F., Marsden, S., Skinner, M. A., Schild, G. C., and Almond, J. W. (1989). Genetic basis of attenuation of the Sabin Type 3 oral poliovirus vaccine. *J. Virol.* **63,** 1338–1344.

World Health Organization. (1990). Requirements for poliomyelitis vaccine (oral). *WHO Tech. Rep. Ser.* **800,** 30–65.